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The Multiple Functions of TRBP, at the Hub of Cell Responses to Viruses, Stress, and Cancer

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INTRODUCTION

Despite the large amount of information obtained from the Human Genome Project, which was completed in 2003, it has become evident that the sequence of our genes is not sufficient to predict the complexity of our cells. Some groups have since turned to the transcriptome, because the expressed genetic material is important, while others have focused on the proteome. More recently, the interactome has been defined as connections between various molecules in the cell with individual molecules acting sequentially or simultaneously in different pathways. With every level of cell biology that we scrutinize, it becomes increasingly apparent that a good understanding of cellular processes requires us to take a step back and look at the outcome of molecular interactions, rather than dissecting a single molecule.

Double-stranded RNA (dsRNA) binding proteins (dsRBPs) play central roles in many cellular processes by bridging RNAs and proteins to form ribonucleoprotein (RNP) complexes (5, 26, 107). Their common feature is the presence of one or several dsRNA binding domains (dsRBDs), which mediate their interaction with dsRNA (6, 21, 37, 75, 135). Most cellular processes require multiple dsRBPs, and individual dsRBPs are involved in multiple pathways. dsRBPs are involved in nearly all aspects of the cellular life cycle, from transcription and translation to development and immunity (56, 96, 118, 129). One dsRBP that has received much attention in the past few years because of its role in RNA interference (RNAi) is the *trans*-activation response (TAR) RNA binding protein (TRBP).

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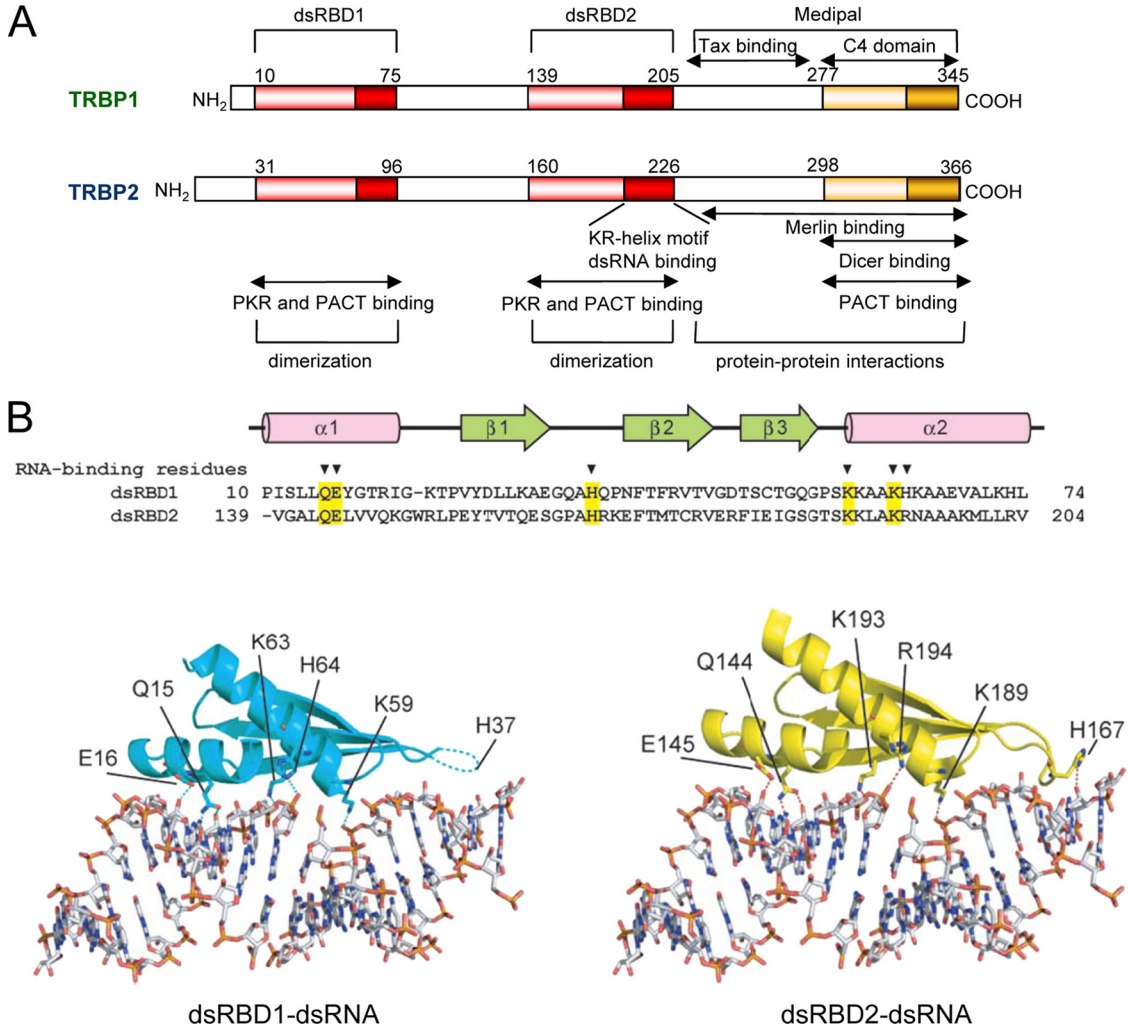

FIG 1 Structural organization of TRBP. (A) Structural organization of TRBP1 and TRBP2. dsRBDs mediate RNA binding and are represented in red. Dark red represents the RNA binding motif, which is fully functional only in dsRBD2 and has been named the KR-helix motif. The Medipal domain is involved in protein-protein interactions. The orange domain (also called C4) has a weak homology with dsRBDs and does not bind RNA but mediates Dicer and PACT interactions. Sites of homo- and heterodimerizations are indicated. The dark red and dark orange domains are basic domains with an α-helix structure. (B) Comparison of the dsRNA binding surfaces of the dsRBDs of TRBPs. (Top) Sequence of dsRBD1 and dsRBD2 of TRBP (amino acid numbers refer to TRBP1). The dsRNA binding residues are indicated with arrowheads. The conserved dsRNA binding residues are shaded in yellow. (Bottom left) Docking model of the TRBP dsRBD1 with dsRNA. The residues with side chains that interact directly with dsRNA are shown in stick representations. The hydrogen bonds are shown by dashed lines. (Bottom right) Structure of the dsRBD2-dsRNA complex. (Adapted from reference 146 with permission of Wiley-Blackwell [copyright 2010 The Protein Society].)

TRBP AS A dsRNA BINDING PROTEIN

Identification

The cDNAs coding for TRBP1 and TRBP2 were cloned based on a protein-RNA binding screen using the highly structured human immunodeficiency virus type 1 (HIV-1) TAR RNA as a probe (52–54). The two proteins are identical except that TRBP2 has 21 additional amino acids at the N-terminal end due to the translation of an alternative first exon (4, 53) (Fig. 1A). TRBP was one of the first proteins involved in the identification of dsRBDs (51, 135). It has two dsRBDs and a C-terminal region called Medipal [Merlin, Dicer, protein kinase R [PKR] activator [PACT] liaison], which mediates protein-protein interactions (31, 40, 51, 84). Analyses of the natural RNA structures bound by TRBP indicated that the protein preferentially binds to double-stranded G-C-rich sequences (51, 89). In the context of synthetic small dsRNAs, TRBP favors asymmetric perfectly matched sequences independently of the sequence length (59, 78, 113).

Structural Domains

TRBP has three structural domains: dsRBD1 is located between amino acids (aa) 31 and 96 in TRBP2, and dsRBD2 is located between aa 160 and 226 (Fig. 1A). The third domain, named the C4 domain (30), is sometimes called half dsRBD or dsRBD type B (aa 298 to 366), because it has some structural homology with dsRBDs in its C-terminal basic portion. However, in TRBP and other dsRBPs, this domain does not bind RNA (66, 68, 135, 145, 146). In contrast, it mediates protein-protein interactions, and the larger region (aa 237 to 366) encompassing C4 is now referred to

as the Medipal region (84). For TRBP-PACT and TRBP-Dicer interactions, the C4 domain is sufficient for binding (30, 84, 135), but a larger region has been defined for the TRBP-Merlin interaction (87). Future refined mapping will determine if the Merlin-interacting region can also be reduced to the C4 domain. In TRBP, dsRBD2 has a stronger dsRNA binding activity than dsRBD1. This difference has been attributed to a 15-aa peptidic sequence within dsRBD2 called the KR-helix motif, which can bind dsRNA by itself (31, 40, 51, 146). The entire protein or the two dsRBDs expressed in tandem have a much higher affinity for dsRNA than either dsRBD alone, indicating that the two domains cooperate for dsRNA binding (31, 51, 146).

Crystal Structure

The crystal structure of dsRBD1 and the solution structure of dsRBD2 have revealed that dsRBD1 and dsRBD2 of TRBP exhibit an α-β-β-β-α fold, which is common to dsRBDs (146) (Fig. 1B). In the context of the KR-helix peptide binding to TAR RNA, each K and R residue is required for RNA interactions (31, 40). For general dsRNA binding by dsRBD1 of TRBP1, the important residues have been mapped to Q15, E16, H37, K59, K63, and H64. The corresponding residues in dsRBD2 (Q144, E145, H167, K189, K193, and R194) are involved in the binding of that domain to dsRNA (146). Mutations of H37, K59, and K63 in dsRBD1 or H167, K189, and K193 in dsRBD2 prevented dsRNA binding (146). In TRBP2, the position numbers are increased by 21 aa. In addition, the thermal stability of dsRBD2 is higher than that of dsRBD1 due to a W residue at position 152 in TRBP1 and at position 173 in TRBP2 (146).

PHYLOGENY OF THE TRBP PROTEIN AND GENE

Evolution and Phylogeny of the TRBP Protein

Following the identification of human TRBP, the cDNA coding for protamine-1 (Prm1) RNA binding protein (PRBP), the murine homolog of TRBP, was cloned from a mouse testis library, and the protein was identified as a key player in translational control during spermatogenesis (16, 89). TRBP2 and PRBP are highly homologous, having 93 to 94% identity and 94 to 96% homology, depending on the subspecies sequenced. With advances in the sequencing of many other organisms, TRBP has been found in several mammalian species with a minimum of 92% identity and no more than 11 gaps (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Fig. 2A). Homologs of TRBP are also found in fish, lizards, crustaceans, insects, and worms, and their functions have been well studied in *Drosophila* sp. (named Loquacious), in *Caenorhabditis elegans* (named RDE-4), and, recently, in shrimps (named Fc- and Mj-TRBP) (14, 44, 105, 113, 143, 144). The identification of a TRBP homolog in tunicates, the closest living invertebrate relative of vertebrates (32), and the presence of both TRBP and a related protein, PACT, in bony fishes suggests that the separation between TRBP and PACT occurred between invertebrates and vertebrates (Fig. 2B). Within vertebrates, birds lack a TRBP homolog but conserve RNAi activity (15, 98), suggesting that the function has been replaced by related proteins.

tarbp2 Gene Characterization

TRBPs and PRBP are encoded by the *tarbp2* gene located on human chromosome 12 and mouse chromosome 15, and one or two pseudogenes, respectively, are present in each species (38, 82, 147). For the human gene, two adjacent promoters initiate the transcription of alternative first exons for TRBP1 and TRBP2 mRNAs. The TRBP1 promoter is upregulated by NF-Y transcription factors, which influences the expression of both isoforms, as observed by the low expression levels of the proteins in astrocytic cells (3, 4). The knockout of the *tarbp2* gene in mice results in a growth defect, and males are sterile, displaying severe oligospermia. In addition, *tarbp2*$^{-/-}$ mice die at weaning, indicating an important role during development (148). In humans, TRBP RNA expression levels are high in the testis and low in the brain (3, 87, 132). This expression profile suggests a particular role for TRBP in undifferentiated cells and cancer cells, as certain testis-associated genes are upregulated in malignant cells (18).

REGULATION OF VIRUSES

Activity in HIV-1 Replication

The first identified function of TRBP was to stimulate the expression of the HIV-1 promoter in human cells, an activity partially linked to the presence of the TAR RNA structure (52). Although HIV-1 is produced at very low levels in murine cells and in human astrocytes, exogenous TRBP enhances both expression from the HIV-1 promoter and viral production in these cells (7, 38, 109). This increased HIV-1 expression level is ascribed to the inhibition of PKR (8, 25, 29) and to a direct activity of TRBP that relieves the translational block caused by the TAR RNA structure (2, 36). The transfection of small interfering RNAs (siRNAs) against TRBP decreases the level of HIV-1 production in HeLa cells, and the transduction of short hairpin RNAs (shRNAs) against TRBP leads to the long-term inhibition of HIV-1 replication in human SupT1 lymphocytic cells, indicating that the protein contributes to viral expression and replication (24, 39), but these findings have not yet been reproduced in primary cells. This property seems to be mediated mainly by a decreased TRBP-PKR interaction and a consequent enhancement of PKR activation, which prevents viral translation, but could also be due to TRBP's interactions with other molecules (24, 109, 127).

Activity in Expression of Other Viruses

TRBP also enhances expression from the early promoter of the simian virus 40 (SV40) and the Visna virus promoter but not the immediate-early promoter from simian cytomegalovirus (52). In the case of the human T-cell leukemia virus type I (HTLV-I) promoter, TRBP increases the basal expression level of the promoter but inhibits Tax-mediated *trans*-activation by direct TRBP-Tax interactions (35, 52). It is currently not clear if the observed effect of TRBP on these promoters occurs directly on transcription or is a translational effect, but the inhibition of Tax has been linked to a nuclear localization signal and Tax binding domain in TRBP located between aa 211 and 269 in TRBP1 (35).

REGULATION OF THE INTERFERON RESPONSE

Binding to PKR

PKR is central in the host innate immune response as an interferon (IFN)-inducible dsRBP that has a serine/threonine kinase activity (48, 102, 125). PKR is activated after binding to low levels of dsRNA through its two dsRBDs and by dimerization. Once active, PKR phosphorylates its substrate, the alpha subunit of eukaryotic translation initiation factor 2 (eIF2α). eIF2α phosphorylation dramatically alters the efficiency and rate of translational

A

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B

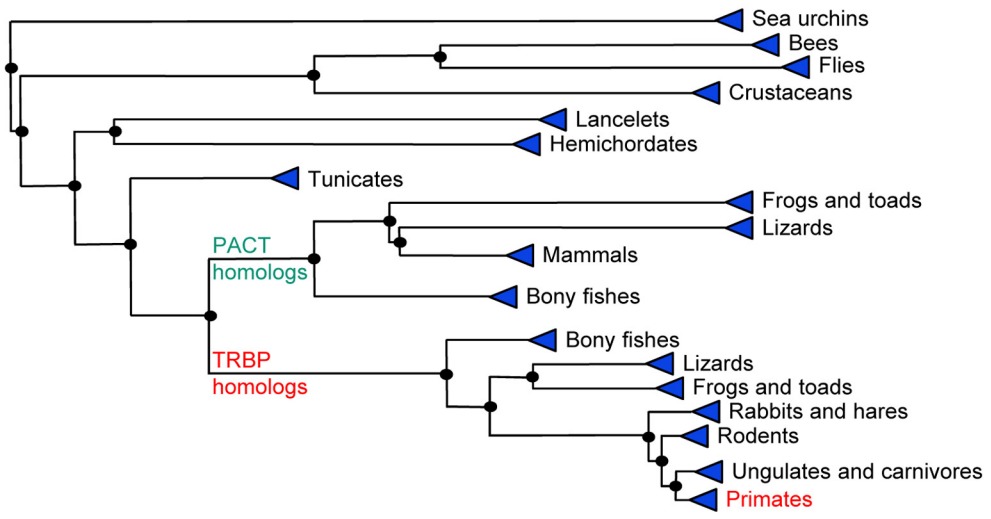

FIG 2 Evolution and phylogeny of TRBP. (A) Homologies between mammalian TRBP2 proteins. Alignments were performed by using BLASTP (http://www.ncbi.nlm.nih.gov/blast/) and COBALT multiple alignments (110). Different amino acids compared to human TRBP2 are shown in red. GenBank accession numbers are indicated on the left. The name of the species used is shown on the right. The human clone in HeLa cells is the first identified protein (52). Mouse PRBP is the first identified murine protein (89). Underlined are dsRBD1 (aa 31 to 96), dsRBD2 (aa 160 to 226), and the C4 domain of Medipal (aa 298 to 366), with amino acid numbers referring to TRBP2. (B) Phylogenetic tree of TRBP2 obtained from COBALT multiple alignments. The phylogenetic tree was created by using the fast minimum-evolution method (33), showing evolutionary distance according to data reported previously by Grishin (61). The branches on the right of the PACT homologs represent proteins that have more homology with PACT than with TRBP2. The branches on the right of the TRBP homologs are more closely related to TRBP2. Proteins between sea urchins and tunicates are related to both PACT and TRBP2.

initiation and is a critical component of antiviral and cell growth pathways (46, 48). In addition to its role in translational regulation in response to dsRNA, several other activators, such as growth factors, cytokines, proinflammatory stimuli, and oxidative stress, also activate PKR. PKR is therefore implicated in the integration and transmission of signals to the translational machinery as well as to factors such as STAT, IFN regulatory factor 1, p53, Jun N-terminal protein kinase (JNK), p38, ATF-3, and NF-κB (48, 121, 125). PKR overexpression decreases—whereas its inhibition increases—the expression levels of several viruses (34, 72, 106, 109, 117). The cellular function of PKR on cell growth appears to be more complicated, because its upregulation or overexpression can inhibit the growth of some tumor cells, whereas an upregulation of PKR has been identified in some malignancies (13, 104, 141). Whether the overall level of PKR is increased or not, its activation is highly regulated by viral and cellular proteins and RNAs (1, 26, 47–49, 56, 117).

The inhibition of PKR activation by TRBP was first identified by its ability to restore the growth of a vaccinia virus lacking the E3L protein and was ascribed to dsRNA sequestration by TRBP (27, 112). Further studies using immunoprecipitation, *in vitro* interactions, and yeast and mammalian two-hybrid assays showed that the two proteins bind directly through their dsRBDs, leading to the inhibition of PKR function (8, 12, 29, 64). Therefore, it is likely that TRBP inhibits PKR function both by dsRNA sequestration and by direct protein-protein interactions.

Consequences of PKR Inhibition by TRBP

A comparison of levels of HIV-1 production in cells producing high or low levels of TRBP demonstrates the consequence of the TRBP inhibition of PKR *in vivo*. HeLa, HEK293T, or lymphocytic T-cell lines efficiently replicate the virus. Astrocytes are brain cells that can be infected with HIV-1 but produce very small amounts of virus (58). Studies using astrocytoma cells showed that there is a major block in the HIV-1 translation of structural proteins due to a heightened PKR activation in these cells after the transfection of an HIV-1 molecular clone (57, 109). This high level of PKR activation correlates with a weak expression of TRBP in these cells compared to that in HeLa cells or lymphocytes and the consequent absence of a PKR-squelching mechanism. The transfection of TRBP is able to partially rescue HIV-1 expression in these cells (25, 109). The TRBP expression level in astrocytes is low because of the weak induction of its promoter. In turn, the poor stimulation of the TRBP1 promoter in astrocytes compared to that in lymphocytes is due to the reduced amount of the NF-Y transcription factor in these cells (3, 4, 109) (Fig. 3A). This model of the consequences of low TRBP expression levels on viral replication in brain cells suggests additional roles for TRBP in the physiological and pathological pathways in which PKR is involved.

DAMPENING OF THE STRESS RESPONSE

PACT and the Stress Response

PACT is a dsRBP that was cloned based on its interaction with PKR in a yeast two-hybrid screen of a human cDNA library using inactive PKR as bait (116). In contrast to the inhibition of PKR by TRBP, mammalian cells transfected with PACT show an enhanced phosphorylation of PKR and eIF2α, which inhibits translation. PACT expressed in bacteria, purified and devoid of any contaminating dsRNA, also activates PKR (116). In contrast to transfection assays that used a truncated PACT at its C-terminal end (68, 116, 119), wild-type (wt) PACT activates the translation of SV40 or HIV promoter-driven luciferase genes, which raises questions about the physiological conditions under which PACT activates PKR (28, 84, 91). Assays using wt and endogenous proteins demonstrated that PACT and PKR activator X (RAX), its murine homolog, are proapoptotic proteins (64, 68, 70, 115, 119) and stress-modulated physiological activators of PKR (28, 115). Stress signals lead to the phosphorylation of PACT on serines 18 and 287, and PACT and RAX need to be phosphorylated to mediate PKR activation (11, 120).

Modulation of the Stress Response by TRBP-PACT Interactions

TRBP and PACT are highly homologous and interact through three domains, the two dsRBDs and the C4 domain in Medipal (79, 84). The stress modulation of PACT activity is explained by its interaction with TRBP under nonstress conditions (28). While the native form of PACT does not constitutively activate PKR in most cells, a 13-aa C-terminally truncated mutant (called PACT305 or PACTΔ13) is able to do so (28, 119). Both forms increase levels of PKR phosphorylation in astrocytes, suggesting that the weak expression of TRBP in these cells plays a role in enhancing the PACT activation of PKR (28, 84). Indeed, PACTΔ13 is a mutant that weakly binds to TRBP and constitutively activates PKR in all cells. In the absence of stress, PKR activation by wt PACT occurs only when there are low levels or an absence of TRBP such as in astrocytes, in cells treated with siRNAs against TRBP, or in *tarbp2*$^{-/-}$ cells (28). PACT activity was also restored by a stress that phosphorylates serine 287 on PACT and disrupts the TRBP-PACT heterodimer, indicating that TRBP controls the PACT activation of PKR by a stress-reversible process (28, 133). Together with TRBP and ADAR1, PACT is involved in the overall regulation of HIV-1 gene expression by PKR during viral replication and may also be involved in other cellular processes (25, 26) (Fig. 3B). The modulation of the TRBP-PACT interaction by stress may also influence several other cellular processes in which both proteins are involved, including RNA interference (RNAi) and oncogenesis.

TRBP FUNCTION IN RNA INTERFERENCE

TRBP in RNA Interference

In addition to its key roles in HIV replication and the regulation of innate immune pathways, in 2005, TRBP was found to be associated with the RNAi machinery (22, 65, 122). RNAi has emerged as a crucial regulatory pathway in all eukaryotic cells and is continually being implicated in new mechanisms of cellular function, proliferation, differentiation, and defense (17, 41, 62, 74, 83, 93, 136). Since RNAi was first described in the nematode *C. elegans* (43), the key proteins of the RNA-induced silencing complex (RISC) have been identified as being Dicer, Argonaute (Ago), and a small dsRNA binding protein. These three proteins are highly conserved among eukaryotes and represent the minimal RISC-loading complex (RLC). In *C. elegans* and mammals, only one isoform of Dicer has been identified and is central to RNAi activity. In *Drosophila melanogaster*, two Dicer proteins have been identified, Dicer-1 and Dicer-2. While Dicer-1 is specialized for the processing of microRNAs (miRNAs), Dicer-2 is involved primarily in the formation of the RISC and in carrying out silencing functions (71). In contrast, two Ago isoforms exist in *Drosophila*

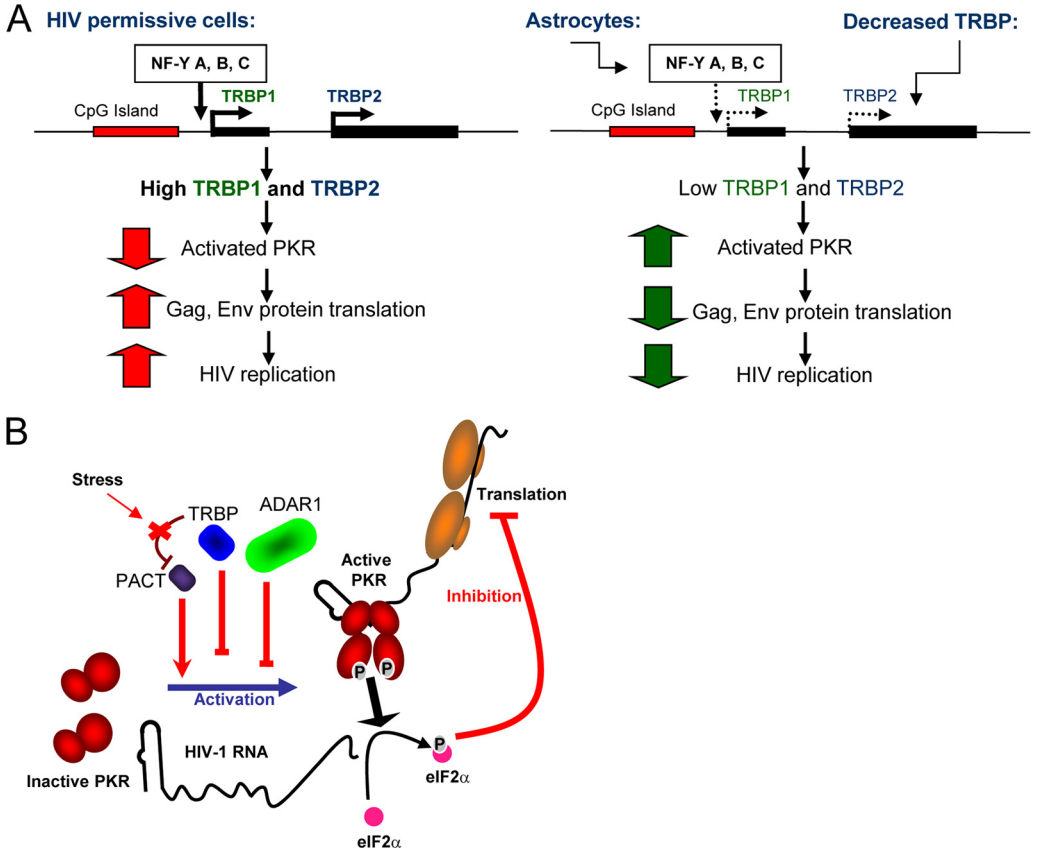

FIG 3 Role of TRBP in regulating HIV-1 expression and replication through PKR regulation. (A) Control of HIV replication in astrocytes compared to HIV-permissive cells through TRBP expression. In HIV-permissive cells, the NF-Y transcription factors activate the TRBP1 promoter, which results in the production of TRBP1 and TRBP2. TRBP binds to PKR and inhibits its activation. This results in the translation of HIV-1 proteins and high-level HIV-1 replication. Astrocytes express low levels of NF-Y transcription factors, which results in low TRBP promoter expression and low TRBP protein production levels. Low TRBP protein expression levels can also be obtained by using siRNAs against TRBP mRNA. Low TRBP expression levels result in high levels of PKR activation, a block of HIV-1 protein production, and low levels of HIV-1 replication (3, 4, 24, 109, 127). (B) Control of PKR activation by TRBP, ADAR1, and PACT during HIV-1 expression. PKR is activated by HIV-1 TAR RNA. This activation is inhibited by TRBP and ADAR1 but enhanced by PACT. TRBP also inhibits PACT activity, which can be restored by oxidative stress (25, 26, 29, 30, 84). (Adapted from reference 25.)

sp., compared to four in mammals and *C. elegans*. While these proteins are highly similar, each isoform appears to have some specificity of function (67, 69). The dsRBPs associated with Ago and Dicer differ from one organism to another. In *C. elegans*, this protein is RDE-4; in *Drosophila*, it is either R2D2 or Loquacious (Loqs), and in humans, it is TRBP (22, 44, 65, 94, 138) (Fig. 4). Although these proteins undoubtedly form a complex with Ago2, as shown by immunoprecipitation (22, 65, 92), it is yet unclear whether TRBP directly binds Ago2 or whether Dicer forms a bridge between the two proteins, as no direct interaction has been shown so far.

Part of the RNA-Induced Silencing Complex

Further studies have demonstrated that *in vitro*, TRBP, Dicer, and Ago2 are able to spontaneously assemble, form the RLC, and carry out RISC activity by processing precursor miRNA (pre-miRNA) and cleaving target mRNAs (85, 97). Whereas two studies suggested that this complex remains intact with multiple rounds of cleavage (60, 90), others indicated that Ago2 is associated more with mature RNA sequences and tends to dissociate after miRNA loading, suggesting that TRBP and Dicer are essential for processing shRNAs/miRNAs but do not participate in target cleavage (63, 97, 99). While few *in vivo* studies have been carried out, results indicate that the presence of TRBP is crucial for RNAi activity. Reconstitution in the yeast *Saccharomyces cerevisiae* showed that Dicer, TRBP, and Ago2 are necessary and sufficient to recapitulate functional human RNAi (137). Cells derived from *tarbp2*$^{-/-}$ mice exhibit virtually no RNAi activity, but this activity is restored by exogenous TRBP (30). In addition, the C4 domain of TRBP, which is crucial for TRBP binding to Dicer, is essential for this restoration of RNAi activity (30). The cellular localizations of TRBP and Dicer indicate a colocalization in the cytoplasm but not in processing bodies, further supporting a major function in RLC formation and miRNA cleavage rather than target cleavage mediated by Ago2 (30, 111).

Role in miRNA Processing

Interestingly, the exact function of TRBP in RNAi remains unclear. While in *C. elegans*, RDE-4 is crucial for the sensing and cleavage of long dsRNA into siRNA, human TRBP appears to act independently of the length of the siRNA (113). TRBP binds to Dicer within the ATPase/helicase domain in a unique domain between the DExH/D and helicase subdomains (30). Interestingly, when this domain is inactivated in Dicer, the protein is unable to

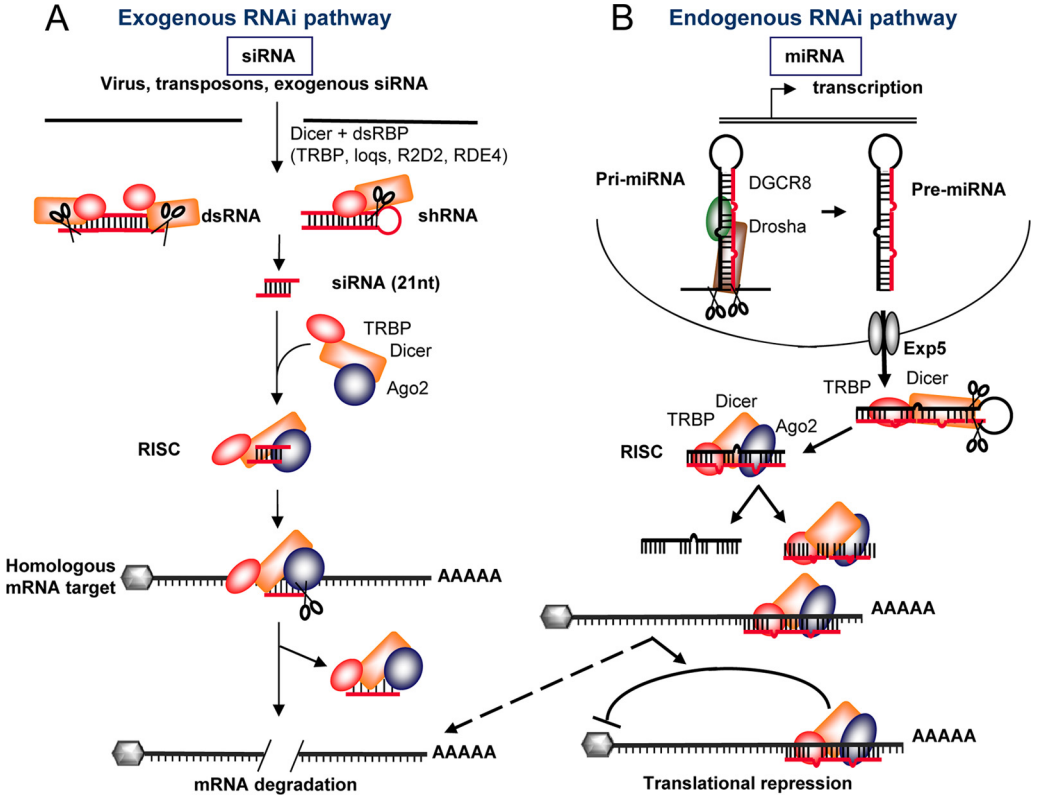

FIG 4 TRBP in the exogenous and endogenous RNAi pathways. (A) Exogenous pathway mediated by siRNAs and shRNAs. Perfectly matched dsRNAs are bound by Dicer and TRBP and cleaved by Dicer to form 21- to 23-nucleotide (nt) siRNAs. The complex then recruits Ago2 and forms the RISC. After strand separation, the complex hybridizes to the complementary sequence in an mRNA. Ago2 mediates the cleavage of the mRNA. (B) Endogenous pathway mediated by miRNAs. Primary mRNAs (pri-miRNAs) are synthesized in the nucleus. They are bound by Drosha and DGCR8. Drosha cleaves them to form the precursor miRNA (pre-miRNA), which is an imperfectly matched dsRNA. Pre-miRNAs are exported to the cytoplasm via exportin5 (Exp5), where they are bound by TRBP and Dicer. Dicer cleaves them to form miRNAs. The RISC is then formed with Ago2. After strand separation, one miRNA strand targets the corresponding mRNA. The translation of this mRNA is generally repressed by a process that involves the recruitment of additional factors and the formation of processing bodies, but in some cases, cleavage of the target mRNA may occur (dotted arrow).

process thermodynamically unstable hairpins (134), whereas when it is deleted or bound to TRBP, Dicer exhibits an enhanced cleavage rate (95). Furthermore, Dicer-TRBP heterodimers are required to sense siRNA asymmetry (108). The two proteins act in concert, and while Dicer is able to preselect effective siRNAs (126), TRBP enhances Dicer-catalyzed miRNA-processing kinetics (20) and acts downstream of siRNA/miRNA production for the selection and incorporation of the guide sequence into the RLC (19, 78, 113). Knockdown studies of TRBP have demonstrated that TRBP contributes to the accumulation of siRNAs and miRNAs and also functions in sensing the target mRNA to effect silencing (19, 22, 65, 90).

Structure of the RISC-Loading Complex

Recently, several groups have examined the structural characteristics of the RLC and have modeled the three-dimensional aspects of the complex from electron microscopy analyses. Dicer, Ago2, and TRBP assemble in a 1:1:1 stoichiometric ratio (97), and the association of TRBP with Dicer yields an "L-shaped" complex (85). Another study identified Dicer alone with an L-shaped structure with a short base. This base represents the DExH/D helicase and ATPase domain of Dicer (142). In agreement with the finding that this domain of Dicer is involved in binding to the TRBP C4 domain (30), when the RLC does not contain TRBP, the Ago-Dicer interaction is weakened, suggesting that TRBP stabilizes the complex. In addition, that study suggested that TRBP contributes to RNAi in several respects. First, the binding of TRBP to Dicer may facilitate the retention of siRNA and may help to orient the binding of Ago2 toward the correct end of the siRNA. Second, TRBP could contribute to the selection of the appropriate strand for loading into the RISC. This hypothesis is further supported by the fact that TRBP recognizes asymmetrical dsRNA molecules better and specifically binds to the 3′ overhang (59).

Relationship between PACT and TRBP in RNAi

Finally, PACT, which has also been recovered in Dicer-containing complexes, contributes to the stabilization and activity of Dicer (79, 90). Although it remains unclear whether TRBP or dsRNA mediates the recruitment of PACT to Dicer, PACT enhances the activity of pre-miRNA processing and the efficiency of siRNA to some extent, and both TRBP and PACT stabilize Dicer (22, 81, 90, 101). In one study, the contribution of PACT was minor, whereas TRBP seemed to be crucial in those assays (90). In another study, PACT contributed to shRNA-mediated silencing but had no influence on siRNA-mediated silencing (79). In contrast, in *tarbp2*$^{-/-}$ cells, where PACT is present, shRNAs were inactive, suggesting that in these cells, PACT cannot replace the RNAi function of TRBP (30). Overall, PACT is found in the 500-kDa com-

plex with TRBP, Dicer, and Ago2 and contributes to the processing by Dicer and the function of miRNAs and shRNAs, but TRBP seems to have the central role, as shown by *in vitro* reconstitution and the lack of functional rescue by PACT in *tarbp2*$^{-/-}$ cells (30, 97). More *in vivo* studies are required to determine if the association between TRBP and Ago2 is maintained during the target RNA cleavage or translation inhibition step in the miRNA process.

ROLE AND REGULATION OF TRBP IN CELL GROWTH AND CANCER

Overexpression and Malignancy

Several lines of evidence indicate that TRBP has a role in promoting cell growth during development and in cancer. Mice in which the *tarbp2* gene has been homozygously disrupted have a growth defect, suggesting an important role during development (148). The overexpression of TRBP in murine cells induces a transformed phenotype, and the injection of these TRBP-overexpressing cells into nude mice induces tumor formation (8). This activity is attributed to PKR inhibition, since similar results were observed with a transdominant, catalytically inactive PKR mutant (80, 103). The growth-promoting function and oncogenicity of TRBP are regulated by a tumor suppressor called Merlin or Schwannomin, encoded by the neurofibromatosis type 2 (*nf2*) gene. Mutations in the *nf2* gene result in brain tumors (123, 140). Multiple protein interactions could explain the role of Merlin as a tumor suppressor (130). Merlin and TRBP interact directly through aa 237 to 366 in TRBP2 and aa 288 to 595 in Merlin, located in their C-terminal ends. Merlin causes TRBP ubiquitination, thereby targeting the protein to the proteasome pathway for degradation (87, 88), and expression levels of TRBP are known to be low in brain tissue (3, 132). This, along with the fact that disruption of Merlin results in brain-specific tumors, suggests that the maintenance of low levels of TRBP is essential for normal brain function and may be mediated by Merlin's activity.

Disruption of the miRNA Pathway

Recent studies shine additional light on the mechanism by which the overexpression of TRBP, its translational modifications, or its mutations contribute to cell growth and cancer-related disorders. Growth induction by TRBP was shown to occur through the mitogen-activated protein (MAP) kinase (MAPK) pathway, which induces the phosphorylation of TRBP by the Erk kinase (114). The phosphorylation of TRBP on serine residues increases the stability of the TRBP-Dicer complex. As a result, certain miRNAs known to promote cell growth, miR-17, miR-20a, and miR-92a, are more expressed, whereas levels of let7 miRNA, which acts as a tumor suppressor, are downregulated. These experiments suggest that a more stable TRBP-Dicer complex promotes cell growth. However, another study found that both wt and phosphomimic TRBPs enhanced Dicer kinetics similarly, suggesting that an increased TRBP-Dicer stability cannot solely be the cause of the observed changes in miRNA expression and cell growth (20).

Truncated TRBP and miRNA Expression

In the colorectal cancer cell line Co115 and in the endometrial cancer cell line SKUT-1B, the deletion or addition, respectively, of one C in the *tarbp2* gene creates a truncated TRBP protein (101). These cells have a severely impaired processing of primary miRNAs into precursors, which results in decreased expression levels of miRNAs with potential tumor suppressor capabilities, such as let7, miR205, miR26a, miR125a, and miR125b. Truncated TRBP is unable to maintain Dicer stability, whereas the wt protein does. The coexpression of the wild-type *tarbp2* gene restores pre-miRNA levels in the cells and reduces the tumorigenicity of the corresponding cells. Although the frequency of these mutations in colorectal cancer has been questioned, their presence was confirmed by another study (50). Taken together, those studies suggest that both impaired TRBP and overexpressed, phosphorylated TRBP promote cell growth by opposite mechanisms, leading to either impaired miRNA processing or decreased miRNA synthesis. Interestingly, in either case, levels of let7 miRNA were significantly decreased. Whether the increased cell growth is due mainly to decreased let7 miRNA levels or whether other mechanisms are involved remains to be determined.

Additional correlations between TRBP expression and mutations have been observed. For example, increased expression levels of miRNAs, Dicer, TRBP, and Ago2 have been shown for prostate cancer cells compared to normal tissue (45). A mutation in the *tarbp2* gene was also found in a gastric carcinoma, a cancer with microsatellite instability and a deregulation of miRNA expression (77). Further studies are needed to confirm the relevance and the role of these mutations in cancer development and to determine if TRBP deregulations contribute to additional cancers. The overexpression, low expression levels, truncations, or mutations of TRBP correlate with cell cycle dysfunction and cancer development, suggesting that an optimal threshold of TRBP seems to be required for appropriate cell growth (Fig. 5).

TRBP-MEDIATED CROSS TALK BETWEEN MULTIPLE PATHWAYS

We have only just started to unravel the mysteries of the cellular interactome. While most functions of TRBP-interacting factors identified in a previous proteomic study remain to be fully characterized (23), some ribonucleoprotein complexes formed around TRBP have revealed important interconnected biological processes. Indeed, by interacting with multiple cellular RNAs and proteins, TRBP bridges multiple pathways that can no longer be considered isolated entities (Table 1). The protein can be associated with different targets at the same time in the cell and mediates cross talk that may affect cell survival (Fig. 6).

From Viral RNA to PKR Inhibition

Although TRBP was originally isolated as a protein that binds a viral RNA, its primary role is not to interact with HIV-1, a virus which likely emerged in humans about a century ago (139). This interaction, which favors HIV-1 replication, quickly emerged as a coevolutionary mechanism in which the virus coopts a cellular function for its own benefit. This benefit was first identified as a mechanism which blocks part of the antiviral innate immune function by binding to PKR (2, 26) but also impacts the general regulation of PKR activation during development and cell growth (48).

Enhancing or Preventing Translation during Development

Murine PRBP/TRBP was first found to repress Prm1 RNA translation by binding to the structured 3′ untranslated region (3′UTR) (89). This activity is required for proper spermatogenesis

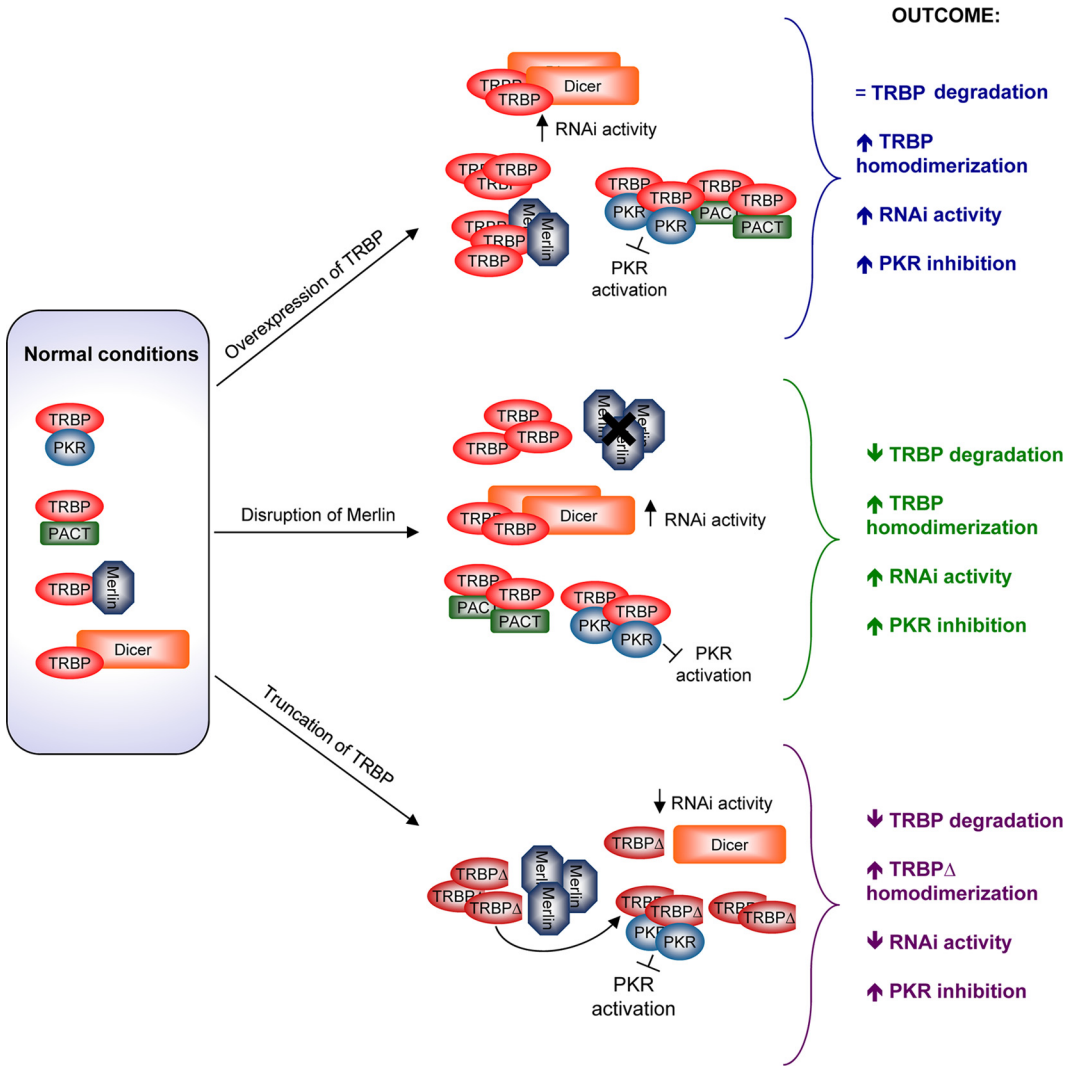

FIG 5 TRBP involvement in malignant phenotypes. (Left) In cells, TRBP can be bound to Merlin, Dicer, PKR, and PACT. These interactions are reversible and regulated. (Top) When TRBP is overexpressed, it binds Dicer for increased RNAi activity, it fully represses PACT and PKR, and it is not fully inactivated by Merlin. This results in a transformed phenotype. (Middle) When the *nf2* gene, coding for Merlin, is disrupted in brain cancers, TRBP becomes overexpressed by a lack of degradation, which results in increased RNAi and increased PKR inhibition. (Bottom) When TRBP is truncated in its C terminus, as seen in some cancer cells, it no longer binds to Dicer, which results in decreased RNAi. It still binds to PKR and inactivates it, but its affinity for PACT is decreased. It no longer interacts with Merlin, which cannot degrade it. As a consequence, TRBP overexpression, Merlin inactivation, or TRBP truncation results in malignant phenotypes by different mechanisms.

during development. The disruption of the *tarbp2* gene causes a growth defect and indicates a role for the protein in enhancing translation (148). At the time when those studies were conducted, TRBP was not known to be part of the RISC. A review of those results suggests that TRBP could enhance translation by inhibiting PKR, unfolding structured RNA, or activating the MAPK pathway, as shown in other studies (29, 36, 114). In a different spatiotemporal context, it could repress the expression of some transcripts when bound to their 3′UTRs as a RISC component (22, 30, 65, 99) or by enhancing the production of specific miRNAs (101, 114). The appropriate expression and the multifunctional activity of TRBP are likely required for normal developmental processes.

From miRNAs to Viral siRNAs

The RNAi pathway is implicated in all aspects of cellular function (83). This has made the role of TRBP at the center of miRNA biogenesis all the more important (22, 30, 65). The dual requirement of TRBP for both HIV-1 replication and RNAi function, together with a possible role of RNAi as an innate immune pathway in mammals (9, 86), does not appear to be compatible (55). In the case of HIV-1, it is clear that TRBP enhances viral expression and replication (7, 8, 24, 25, 29, 38, 39, 52, 109, 127), which suggests that in this context, TRBP may be more frequently associated with the PKR ribonucleoprotein complex and the TAR RNA (2, 26) than with the RISC. Alternatively, HIV-1 might use cellular miRNAs and the RISC to enhance its own replication, as does hepatitis C virus (24, 55, 73). The characterization of HIV-1 TAR RNA as an RNAi suppressor, which sequesters TRBP and prevents the virus from being cleaved by RNAi, increases the difficulty of reconciling the TRBP enhancement of HIV-1 replication and the role of RNAi as a defense mechanism against the virus (10). In

TABLE 1 Molecular partners and inhibitors of TRBP

Molecular partner or inhibitor	Activity of TRBP	Reference(s)
Cellular partner		
TRBP	Forms homodimers	84
PACT	Sequesters PACT and prevents PKR activation	28, 79, 84, 133
PKR	Inhibits PKR activation	8, 12, 29, 64
Dicer	Acts as a cofactor for RNAi	22, 30, 65
Merlin	Is a substrate for ubiquitination	87, 88
siRNA/shRNA/miRNA	Loads small RNAs into the RISC	19, 20, 78, 108, 113
Viral factor		
HTLV-I Tax	Inhibits Tax-mediated transactivation of the LTR[a]	35, 41
Ebola virus VP35	Is a target of VP35-mediated RNAi suppression	42
HIV-1 TAR	Alleviates translational block of mRNA and is a target of TAR-mediated RNAi suppression	52-54
vsiRNA/vmiRNA	Loads small RNAs into the RISC	19, 20, 78, 108, 113
Synthetic compound		
Enoxacin	Is a substrate of enoxacin-mediated enhancement of miRNA activity	100
siRNA/shRNA targeting TRBP	Is a target of RNAi and results in decreased function	22, 24, 39, 65

[a] LTR, long terminal repeat.

contrast, Ebola virus limits RNAi efficiency, and TRBP is a direct target of the viral VP35 protein (42). Indeed, VP35 acts as an RNAi suppressor by directly or indirectly binding to TRBP, suggesting that this interaction prevents RISC activity. Several mammalian viruses, mostly herpesviruses, express viral siRNAs (vsiRNAs), which control their own expression. In the case of herpesviruses, these vsiRNAs regulate the viral life cycle by allowing a switch between lytic and latent cycles, but the role of TRBP in this switch is not known (62). The effect of the overexpression or inhibition of TRBP has not been studied for other viral infections besides HIV. These assays would give insight into the role of TRBP-mediated cross talk between different pathways during various viral infections. It would also help to resolve the issue of the controversial debate on innate immunity mediated by RNAi in response to viral infections in mammals (62, 128).

Cooperation or Competition between TRBP and PACT?

TRBP and PACT have evolved from a common ancestor in invertebrates and have separated in vertebrates (Fig. 2B). They have the same protein organization and are highly homologous. Because of this similarity, they have been proposed to be interchangeable in development and RNAi but to have opposite functions in regard to PKR activation (64, 79, 90). However, their functional similarities or differences are not easy to evaluate considering that the two proteins bind to each other and regulate each other's functions (28, 79, 84, 133). In the context of PKR regulation, it now seems clear that the two proteins have opposite functions due to their C4 domains, but PACT can exert its PKR-activating function only in the absence of TRBP or under stress conditions, which dissociates the interaction between the two proteins (28, 133). Within the RISC, whether PACT can replace TRBP as a partner for Dicer and reconstitute the RLC is still under debate. Some groups, but not all, have found PACT to be associated with the RISC (79, 90). Furthermore, it has not been determined whether PACT interacts with Dicer directly or through TRBP or dsRNA, and so far, no *in vitro* reconstitution has been shown solely with PACT, Dicer, and Ago2. The idea of interchangeability between TRBP and PACT arose from the fact that both *tarbp2*$^{-/-}$ and *prkra*$^{-/-}$ (PACT knockout) mice are viable, with defects in spermatogenesis and ear development, respectively (124, 148). Considering that TRBP is involved in so many different processes, it seems surprising that TRBP knockout mice could survive, if only until weaning. In addition, TRBP knockout cells derived from these mice, which exhibit no RNAi activity (30), survive extremely well in cell cultures and become immortalized in the absence of any transforming agent (our unpublished observations). However, the fact that *tarbp2*$^{-/-}$ mice die at weaning demonstrates that the proposed compensatory effect of PACT is only partial during development and not sufficient to allow survival. These remaining questions indicate that more *in vitro* and *in vivo* studies need to be done to determine if PACT is an essential protein, a TRBP substitute, or a contributing protein that cooperates or competes with TRBP within the RISC and during development.

Combining miRNA Disruption and PKR Inhibition in Cancer

The association of either the overexpression, modification, or mutation of TRBP with several types of cancer raises important questions about TRBP-mediated oncogenesis. The inhibition of PKR by TRBP contributes to tumorigenesis in mice (8), and phosphorylated TRBP contributes to accelerated cell growth following the activation of the MAPK pathway (114). In addition, mutations of TRBP that inhibit its binding to Dicer and Merlin lead to a disruption of the miRNA pathway (30, 87, 101). These mechanisms are not exclusive and likely contribute to cell growth control during normal development and to oncogenesis when they are disturbed or uncontrolled. Furthermore, the involvement of TRBP and Ago1 in the transcriptional gene silencing of the RASSF1A promoter, which is epigenetically silenced in cancer, may lead to the discovery of additional roles of TRBP in cancer cells (76). More studies of these mechanisms could show a more important role of TRBP in a larger variety of cancers. The potential of small molecules to inhibit cancer cell growth by binding TRBP and restoring a normal miRNA pathway might also have an impact on PKR activation in these cells (100, 131).

CONCLUSIONS AND PERSPECTIVES

The dsRBP TRBP has emerged as an essential protein. It is critical for the survival of the entire organism and is heavily involved in

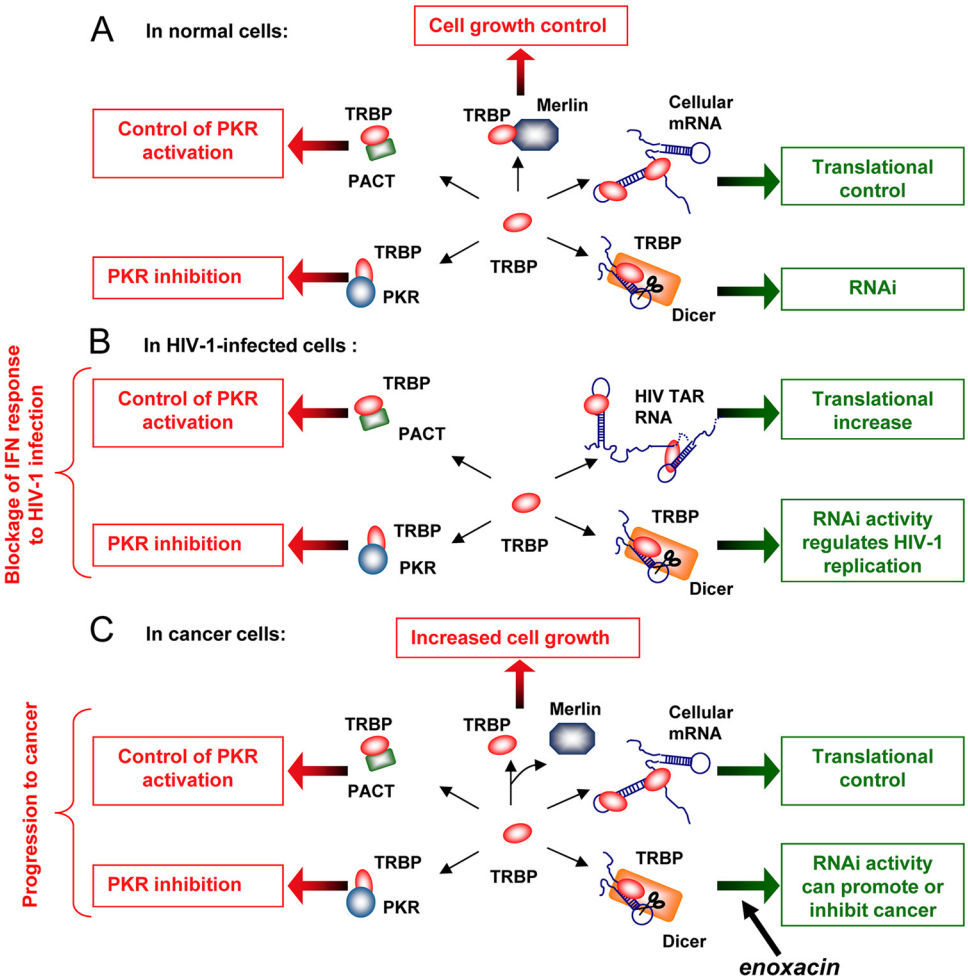

FIG 6 Known and possible interactions and functions of TRBP under normal conditions, in HIV-infected cells, and in cancer cells. (A) Known and possible interactions and functions of TRBP under normal conditions. TRBP interacts with cellular RNAs and miRNAs as well as with Dicer, PKR, and PACT to regulate translation, RNAi, and PKR activation. Its binding to Merlin mediates its degradation and control of cell growth. (B) Known and possible interactions and functions of TRBP in HIV-1-infected cells. TRBP interacts with HIV-1 TAR RNA to increase the translation of HIV-1 mRNAs, while it still binds to cellular mRNAs and miRNAs. TRBP binds to Dicer, PKR, and PACT to regulate RNAi and PKR activation. Its interaction with Merlin has not been tested in infected cells. (C) Known and possible interactions and functions of TRBP in cancer cells. The disruption between TRBP and Merlin leads to an increased TRBP concentration and increased cell growth. The overexpression or truncation of TRBP results in increased binding to cellular mRNA and miRNA, which disturbs translational control. Increased or decreased binding to Dicer may disturb RNAi activity and lead to cancer. The small molecule enoxacin enhances miRNA processing by TRBP and inhibits cancer growth (100). Increased binding to PKR and PACT inhibits PKR activation.

spermatogenesis and cell growth. More specifically, it has been shown to be a crucial regulator of many cellular functions: TRBP is involved in the regulation of the IFN pathway, is essential for HIV replication, has a function in the stress response by sequestering PACT, is an essential component of the RNAi machinery, and contributes to cell growth and cancer. In most of these pathways, TRBP is a hub of interactions connecting various pathways in cell growth and in cellular responses to infection. Some of these interactions have been well characterized, but many more have been recently identified and need to be functionally characterized (23). The comparison between the proteins and RNAs that interact with TRBP in normal and pathological contexts will give further insight into the various cellular functions of this multifunctional protein.

The plurifunctionality of TRBP may have important implications for therapies targeting TRBP. On the one hand, the knocking down of TRBP may be extremely beneficial in situations of infections with oncogenic viruses, as TRBP is involved both in dampening the antiviral response and in promoting oncogenesis. On the other hand, the knocking down of TRBP expression in cells is likely to disrupt more than one pathway, which may impede cellular survival, and this may not be desirable in all circumstances. The identification of a molecule that specifically binds TRBP and restores a normal miRNA profile in cancer cells may help to elucidate the role of TRBP in other pathways and to explain the downregulation of certain miRNAs but not others (100, 131). Further elucidation of these pathways will help to identify therapies to target TRBP and restore normal pathways that have become dysfunctional in various pathologies.

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